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Introduction of tag epitopes in the inter-AUG region of foot and mouth disease virus: Effect on the L protein

Maria E. Piccone[a,b,*], Fayna Diaz-San Segundo[a,c], Edward Kramer[a], Luis L. Rodriguez[a], Teresa de los Santos[a]

[a] Plum Island Animal Disease Center, ARS, USDA, Greenport, NY, United States
[b] Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT, United States
[c] Oak Ridge Institute for Science and Education, PIADC Research Program, Oak Ridge, TN, United States

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ABSTRACT

Foot-and-mouth disease virus (FMDV) initiates translation from two in-frame AUG codons producing two forms of the leader (L) proteinase, Lab (starting at the first AUG) and Lb (starting at second AUG). In a previous study, we have demonstrated that a cDNA-derived mutant FMDV (A24-L1123) containing a 57-nucleotide transposon (tn) insertion between the two AUG initiation codons (inter-AUG region) was completely attenuated in cattle, suggesting that this region is involved in viral pathogenesis. To investigate the potential role of the Lab protein in attenuation, we have introduced two epitope tags (Flag: DYKDDDK and HA: YPYDVPDYA) or a small tetracysteine motif (tc: CCGPCC) into the pA24-L1123 infectious DNA clone. Mutant viruses with a small plaque phenotype similar to the parental A24-L1123 were recovered after transfection of constructs encoding the Flag tag and the tc motif. However, expression of the Flag- or tc-tagged Lab protein was abolished or greatly diminished in these viruses. Interestingly, the A24-L1123/Flag virus acquired an extra base in the inter-AUG region that resulted in new AUG codons in-frame with the second AUG, and produced a larger Lb protein. This N terminal extension of the Lb protein in mutant A24-L1123/Flag did not affect virus viability or L functions in cell culture.

1. Introduction

Foot-and-mouth disease virus (FMDV) belongs to the genus *Aphthovirus* of the family *Picornaviridae* and exists as seven distinct serotypes throughout the world. The FMDV genome is a single-stranded RNA molecule of approximately 8500 nucleotides (nt) and translates as a large polyprotein which is processed to yield three viral precursors, P1-2A, P2 and P3, by autoproteolytic cleavage (Grubman and Baxt, 2004). At the amino terminus of the viral polyprotein is the leader (L) protein of 199–202 amino acids, depending on the viral serotype (Carrillo et al., 2005; van Rensburg et al., 2002). L is a papain-like proteinase that removes itself from the viral polyprotein (Kleina and Grubman, 1992; Piccone et al., 1995b; Roberts and Belsham, 1995) and also cleaves the eukaryotic translation initiation factor 4G (eIF4G), resulting in the shutoff of the cap-dependent host protein synthesis, without affecting cap-independent viral protein synthesis (Devaney et al., 1988; Kirchweger et al., 1994). In addition, L plays a role in FMDV virulence by regulating the host innate immune responses (Chinsangaram et al., 1999; de los Santos et al., 2006, 2007, 2009).

The leader protein is produced in two forms, Lab and Lb, through the initiation of translation at two in-frame AUG codons located 84 nt apart (inter-AUG region). These two AUG codons have been shown to be present in viruses of all seven serotypes (Carrillo et al., 2005; Sangar et al., 1987). The smaller protein (Lb) is initiated from the second functional AUG and is synthesized in excess with respect to Lab despite its downstream location, both in *in vitro* translation reactions and in infected cells (Clarke et al., 1985; Sangar et al., 1987). Previous investigations have shown that the relative use of each initiation site is dependent on its surrounding nucleotide sequence and the RNA structure upstream and downstream of the initiation AUG (Belsham, 1992; Lopez de Quinto and Martinez-Salas, 1998; Ohlmann and Jackson, 1999). In addition, it has been demonstrated that the presence of the second AUG initiation codon, but not the first one, is essential for virus replication (Cao et al., 1995). Both forms of the L protein possess similar proteolytic activities (Medina et al., 1993; Strebel et al., 1986), and therefore it is not known why the two forms of L are synthesized during the FMDV infection. Notably, the L protein exhibits

Abbreviations: FMDV, foot-and-mouth disease virus; L, leader protein; HA, influenza-hemagglutinin; tc, tetracysteine motif; NCR, non coding region; SAT, South African territories.

* Corresponding author at: Plum Island Animal Disease Center, P.O. Box 848, Greenport, NY 11944-0848, United States. Tel.: +1 631 323 3102, fax: +1 631 323 3006.
E-mail address: maria.piccone@ars.usda.gov (M.E. Piccone).

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variability comparable to that of the structural proteins, the most variable being the inter-AUG region (Tosh et al., 2004; van Rensburg et al., 2002).

We have recently shown that the inter-AUG region can tolerate insertions of heterologous sequences. Importantly, mutant viruses carrying a 57 nt transposon (tn) insertion within that region replicated more slowly than the wild type (WT) virus, and were highly attenuated in bovines (Piccone et al., 2010). The tn insertion did not interrupt the polyprotein reading-frame of these viruses, but the amino acid composition of the N terminus of the L protein was profoundly altered. It is possible that these changes accounted for the attenuation phenotype.

To date, no studies have been reported addressing the individual role of each form of L in virus replication and pathogenesis. With the aim of differentiating the individual forms of L, we have introduced two "epitope tags" (HA and Flag) and a small tetracysteine (tc) motif in the inter-AUG region of a tn-containing virus. The HA and Flag tags are particularly useful for immunoreactive experiments. On the other hand, the tc motif has been very valuable in visualizing many dynamic processes in living cells (Arhel and Charneau, 2009; Das et al., 2009; Perlman and Resh, 2006; Rudner et al., 2005), and could allow visualization of Lab within infected cells by fluorescence microscopy. Here, we report the characterization of the recovered L-mutant viruses and the effect of the tags on L functions. We discuss these results with respect to the mechanism of attenuation of the previously reported tn containing mutant viruses.

2. Materials and methods

2.1. Cells, plasmid constructs and mutant viruses

BHK-21 αVβ6 cells (Duque et al., 2004) were used to propagate virus and BHK-21, bovine kidney cell line (LF-BK) (Swaney, 1988) and secondary lamb kidney (LK) cells (House and House, 1989) were used for titration, *in vitro* growth and plaque assays. Flag (DYKDDDK), influenza-hemagglutinin (HA) (YPYDVPDYA), or a small tetracysteine motif (tc) (CCPCC) tags were introduced at the unique NotI restriction site of previously constructed mutant plasmid pA24-L1123. This plasmid derives from the infectious clone pA24-WT and contains a 57 nt transposon (tn) insertion in the inter-AUG region (Piccone et al., 2010). Briefly, pA24-L1123 was used as template in a PCR amplification using synthetic oligos: (sense) 5′-CACTTTATTGCGGCCGCA**GACTAT AAGGACGATGATGACAAG**AGATGTGTACAAGAGACAGCTATC-3′ (Flag, bold case); 5′-CACTTTATTGCGGCCGCA**TACCCATACGATGTTCCAGATTACGCT** AGATGTGTACAAGAGACAGCTATC-3′ (HA, bold case) or 5′-CACTTTATTGCGGCCGCA**TGTTGTGGCCCTTGTTGT**AGATGTGTACAAGAGAC-3′ (tc, bold case) and antisense oligo nt 2924—5′-CTTTGAATCAGTGGAACCTG-3′ on the unique SpeI site of the pA24-WT plasmid. The PCR products were digested with NotI and SpeI and cloned between the NotI and SpeI sites of pA24-L1123. All the tagged mutant plasmids were sequenced to confirm the presence of the tag using an Applied Biosystems 3730 xl automated DNA sequencer (Applied Biosystems, Foster City, CA).

RNA derived from pA24-WT and pA24-L tag mutants were transfected into BHK-21 cells by electroporation as described previously (Mason et al., 1994). Viruses recovered from these transfections were passed three times in BHK-21 αVβ6 and virus stocks were prepared and sequenced.

2.2. In vitro transcription and translations assays

To generate RNA for translation assays, L mutant plasmids and parental pA24-WT were linearized with SpeI, and plasmid pL (encoding the L gene containing both AUGs) with BamHI. Transcription was performed using the MegaScript T7 kit (Ambion, Austin, TX) as recommended by the manufacturer. For the transcription of the pL control, the reactions were performed in the presence of the cap analog [m7G(5′)ppp(5′)G].

In vitro translation assays were carried out using rabbit reticulocyte lysate (Promega, Fitchburg, WI) as described by Piccone et al. (2010). Translation products were separated by gradient 10–20% SDS-PAGE and detected by autoradiography.

2.3. Analysis of growth of L tagged FMD mutant viruses

Mutant viruses were characterized by plaque assays in BHK-21, LK (House and House, 1989) and LF-BK cells (Swaney, 1988). Plaques were visualized under a gum tragacanth overlay stained at 48 h post-infection (hpi). One-step growth curves were performed in LF-BK cells. Briefly, cells were infected at a multiplicity of infection (MOI) of 10 at 37 °C. After 1 h adsorption, cells were rinsed with 150 mM NaCl 20 mM morpholineethanesulfonic acid (MES) pH 6 to inactivate unadsorbed virus and incubated at 37 °C. At 4, 8 and 24 hpi, cells were harvested and virus titrated on BHK-21 cells as described previously (Piccone et al., 1995a).

2.4. Western blot analysis

Cell lysates from infected LF-BK cells were prepared and fractioned on a 12% SDS-PAGE. Proteins were transferred to PVDF membranes and probed with rabbit polyclonal anti-leader and anti-p220 antibodies (kindly provided by Dr. Grubman), anti-NF-κB-p65/RelA (Ab-1 RB-1638, NeoMarkers, Lab Vision, Freemont, CA), anti-VP1 (6HC4 (Baxt et al., 1984)), and anti-tubulin-α (Ab-2 MS-581 Clone DM1A, NeoMarkers, Lab Vision). For detection, an Immun-Star™ HRP chemiluminescent kit (Biorad, Hercules, CA) was used following the manufacturer's directions.

2.5. Immunofluorescence microscopy

Viral proteins were detected in infected cell cultures by indirect immunofluorescence microscopy as described previously by de los Santos et al. (2007). Briefly, LF-BK cells grown on glass coverslips were infected with A24-WT or L tagged mutant viruses at MOI 10. At different hpi, cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100® (Sigma) in PBS, blocked with blocking buffer (PBS, 2% bovine serum albumin [BSA], 5% normal goat serum, 10 mM glycine) and immunostained using antibodies against leader (rabbit polyclonal) or structural protein VP1 (6HC4 (Baxt et al., 1984)). Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes, Invitrogen, Carlsbad, CA) conjugated secondary antibodies were used for detection. Nuclei were visualized by DAPI staining included in ProLong Gold Antifade mounting media (Invitrogen). Cells were examined in an Olympus BX40 fluorescence microscope and pictures were taken with DP-70 digital camera/DP-BSW v2.2 software (Olympus America, Central Valley, PA).

3. Results

3.1. Generation and characterization of L tagged FMD mutant viruses

In the absence of reagents that allow differentiating between the Lab and Lb proteins, we introduced a tag sequence in the inter-AUG region of a previously constructed mutant plasmid pA24-L1123 (Piccone et al., 2010). As shown in Fig. 1A, three different tag sequences (Flag, HA and tc) were inserted into the unique NotI site contained in the tn insertion of pA24-L1123.

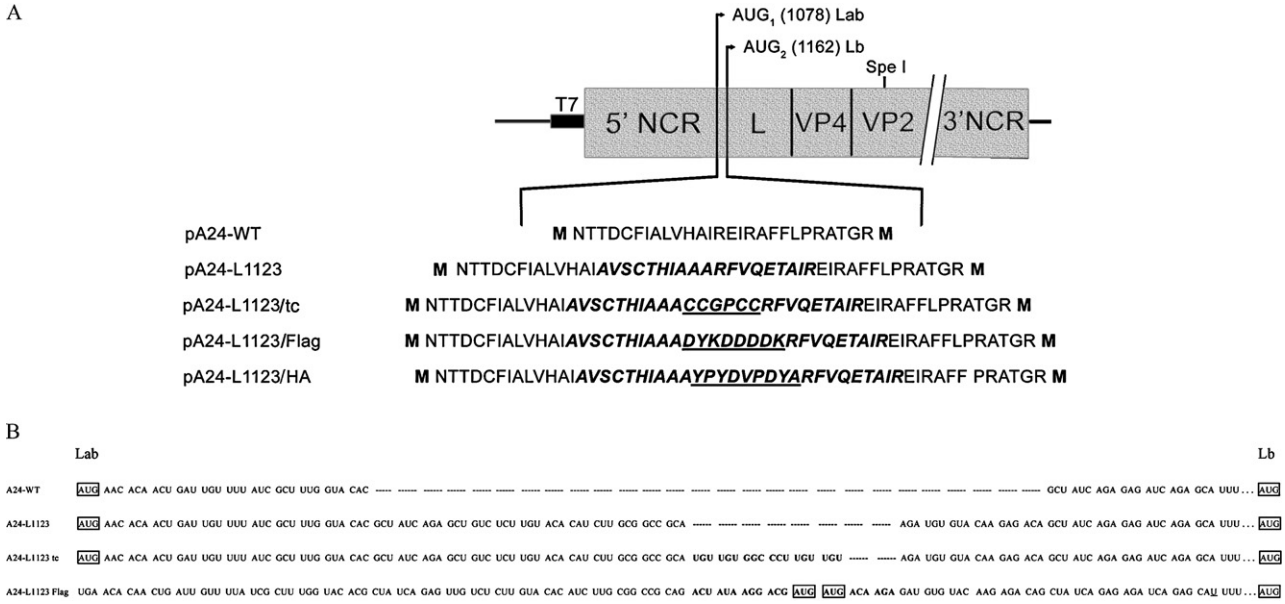

Fig. 1. (A) Schematic diagram of the FMDV genome and plasmids used in this study. The FMDV full-length genome cDNAs in these plasmids derived from the infectious cDNA clone, pA24-WT, were preceded by a synthetic T7 RNA polymerase promoter. The AUG codons at positions 1078 and 1162, corresponding to the two initiation sites of FMDV type A24 (accession number AY593768) are shown. Mutant plasmid pA24-L1123 was produced by transposon (tn) insertion mutagenesis of pA24-WT and contains an insertion of 19 amino acids indicated in italic and bold at position 1123. Leader-tag proteins were constructed by inserting various tag sequences in the NotI restriction site present in the tn insertion of pA24-L1123. Amino acid sequences of the tag are underlined. Unique SpeI restriction site is indicated. NCR: non-coding region. (B) Nucleotide sequence analysis of the L-tagged virus recovered. Alignment of partial sequences of the region located between the first and the second AUG initiation codons of A24-WT and L mutant viruses. AUG initiation codons corresponding to Lab, Lb as well as potential initiation sites in the A24-L1123/Flag mutant are boxed. The nucleotide sequence of each tag is indicated in bold. The position of the extra U is underlined. (–) indicates an absent sequence.

Viable viruses were obtained from cells transfected with RNA derived from the constructs containing the Flag and the tc tag after BHK-21 αVβ6 cell passages. Mutant virus A24-L1123/Flag showed a cytophatic effect (CPE) after three passages, whereas mutant A24-L1123/tc showed an extensive CPE at 24 hpi in passage 2. In contrast, we were not able to recover viruses from the HA tagged constructs even after several attempts, probably indicating that the nucleotide sequence (or derived amino acid sequence) of the HA tag was not compatible with the generation of viable virus. Sequence analysis of the rescued viruses revealed the presence of the expected tag sequence for the A24-L1123/tc mutant, while the A24-L1123/Flag mutant contained an extra base in the Flag insertion. Due to this insertion, the second AUG was out-of-frame with respect to the first AUG codon, but two new adjacent AUG codons in-frame with the second AUG codon appeared at positions −78 and −75 (Fig. 1B).

We then examined the mutant viruses by their plaque sizes and growth characteristic in cell cultures. Side-by-side comparison of plaques in BHK-21, LF-BK and LK cells showed that both A24 L tag mutant viruses formed plaques smaller than those formed by WT virus, but similar to those of attenuated mutant virus A24-L1123 (Piccone et al., 2010) (Fig. 2A). A single-step growth kinetics in LF-BK cells revealed no significant differences between the viruses, although the overall yields of the L mutant viruses were lower than for A24-WT (Fig. 2B).

3.2. Effect of the tags in self-processing of L

To test the ability of the tagged L proteins to self remove from its precursors, pA24-WT and pA24-L mutant plasmids were digested with SpeI, which cleaves within the structural protein VP2 (position 2512) and removes the downstream portion of the viral genome from the plasmids allowing a clearer interpretation of the viral protein pattern in the SDS-PAGE. T7 RNA transcripts of SpeI-digested pA24-WT, pA24-L1123 and pA24-L tag mutants, as well as transcript of control plasmid pL, were translated *in vitro* in rabbit reticulocyte lysates. Fig. 3 shows that all synthesized L mutant proteins were able to cleave themselves from the precursor L-Vp4-Vp2′ and that the Lb band was absent in all L mutant translation reactions. We were surprised to find several bands present in the mutant translation products, since no other viral proteins of similar size were expected. Moreover, all these products were immunoprecipitated by an anti-L antiserum (data not shown). It should be noted that proteins synthesized in a rabbit reticulocyte lysate do not necessarily reflect the viral proteins produced during an infection in cell culture or *in vivo*.

With the exception of pA24-L1123/Flag, all mutant translation mixes showed a profile similar to that of the parental pA24-L1123, including a band that was also present in the WT translation mix. A unique larger L product was observed in the Flag-tagged L plasmid, consistent with initiation of translation at the first AUG and containing the Flag tag (lane 4). However, this protein was not immunoprecipitated by an anti-Flag antiserum (data not shown), suggesting that initiation occurred at an alternative initiation codon contained within the insertion and disrupting the proper Flag amino acid composition. Similarly, translation products derived from pA24-L1123/HA showed a faint higher molecular weight band.

These results indicate that the presence of the tn insertion in these mutant L plasmids forces translation initiation *in vitro* from other than the second AUG codon since no Lb was detected.

3.3. Expression of functional tagged L proteins in infected cells

The WT and mutant L proteins expressed in LF-BK cells were detected by western blotting analysis using a rabbit polyclonal anti-leader antibody (Fig. 4). Because the L mutant viruses showed different growth rates as compared to the WT virus in these cells (see Fig. 2B), we used higher multiplicities of infection in order to have comparable amounts of protein.

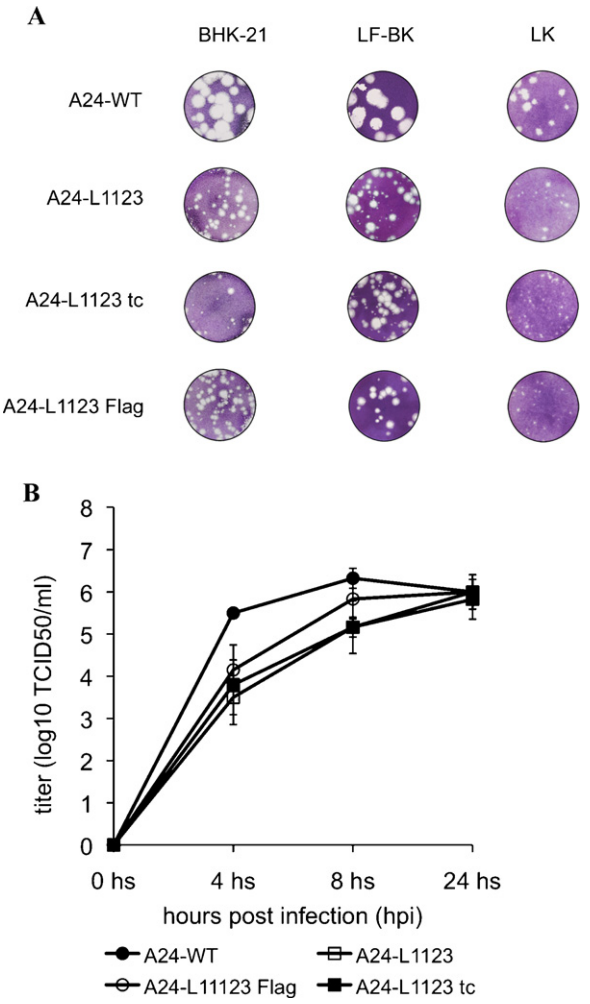

Fig. 2. (A) Plaque morphology of A24-WT and A24-L mutant viruses in different cells. Viruses were plaque-assayed under tragacanth gum on BHK-21, LF-BK and LK cells and cells were stained at 48 h post-infection (hpi). Plaques from appropriate dilutions are shown. (B) Growth of FMDV L-mutants in cell culture. LF-BK cells were infected with WT or L-mutant viruses at a multiplicity of infection (MOI) of 10. At 0, 4, 8 and 24 hpi, cells and supernatants were harvested and the viral titers were determined by TCID50/ml on BHK-21 cells. The error bars indicate the standard deviation. The values of the viral titer represent the average obtained from triplicate experiments.

Results revealed that mutant viruses A24-L1123 and A24-L1123/tc produced mainly Lb protein (lanes 2 and 3 respectively), indicating that the insertions did not interfere with initiation from the second AUG codon. For A24-WT, a band of higher molecular weight was detected, suggesting translation initiation from the first AUG (Lab). Minor bands of slower mobility were also observed for the L mutant viruses, suggesting that L proteins were translated from the first AUG and contained the amino acid sequences encoded by the tn and the tc motif. However, the nature and the origin of the product of intermediate size in the A24-L1123/tc lane was unclear, since – based on their apparent size – there were no AUG codons between the tc tag and the second AUG codon. Interestingly, A24-L1123/Flag virus (lane 4) showed an L protein slightly bigger than Lb, consistent with initiation of translation from a new AUG acquired in the inter-AUG region during viral growth, as shown in Fig. 1B.

We have previously demonstrated that Lb translocates to the nucleus of infected cells and regulates the activity of transcription nuclear factor NFκB (de los Santos et al., 2007). To investigate if viruses expressing the extended L protein are able of processing cellular proteins in a manner similar to that of the WT virus, we examined the pattern of NFκB protein in infected cell extracts. Western blot analysis showed that the p65 subunit of NFκB disappeared during the infection by either WT or L mutant viruses (Fig. 5).

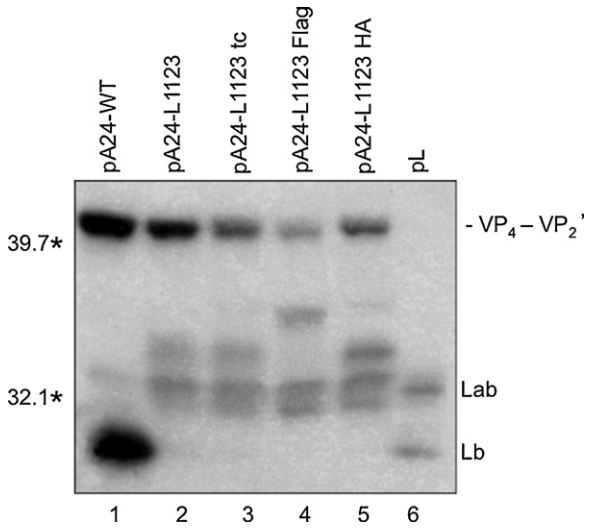

Fig. 3. *In vitro* translation products of WT and L-mutant plasmid RNA transcripts. Transcripts derived from plasmids pA24-WT (lane 1) and pA24-L mutants (lanes 2–5) linearized by restriction enzyme SpeI were used to program protein synthesis in rabbit reticulocyte lysates. The (^{35}S) methionine-labeled proteins were analyzed by SDS-PAGE (10–20% acrylamide gradient) and autoradiography. Lane 6 shows translation products of transcripts derived from a plasmid (pL) encoding for the L gene and containing both AUG initiation codons under the control of a T7 RNA polymerase promoter. Molecular weight markers and cleavage products are indicated on the left and right of the panel respectively.

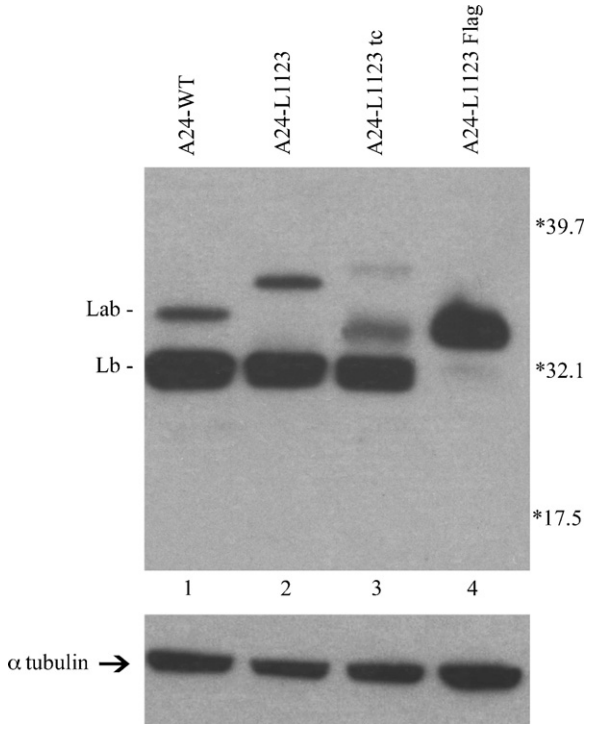

Fig. 4. Expression of the leader protein in LF-BK cells infected with L-mutant viruses. LF-BK cells were infected with wild-type virus A24-WT (lane 1) and the L-mutant viruses (lanes 2–4) at low MOI (0.0001 and 0.01 respectively) and cytoplasmic cell extracts were prepared after 24 hpi. Equal amounts of cell extracts were separated by 12% SDS-PAGE, blotted, and probed with a rabbit polyclonal anti-leader antibody (top) or mouse monoclonal anti-tubulin antibody as a loading control (bottom). Protein size markers (kDa) are indicated on the right.

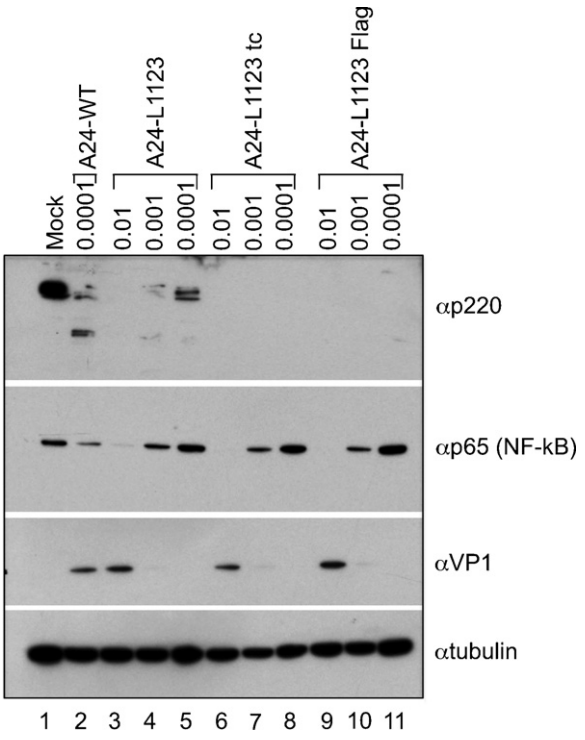

Fig. 5. Processing of cellular proteins during infection with WT and L-mutant viruses. LF-BK cells were infected with A24-WT (lane 2) and L-mutant viruses at the indicated MOIs (lanes 3–11). At 24 hpi, cytoplasmic cell extracts were prepared and analyzed by western blotting using anti-p220, anti-NFκB, anti-VP1 and anti-tubulin antibodies.

Similarly, cleavage of the translation factor eIF-4G (p220) was basically complete when comparable amounts of structural protein VP1 were present in the cell lysates (Fig. 5). These results are in agreement with those reported by Strebel et al. (1986) indicating that the proteolytic function of L is not affected by an N extension of the protein.

Finally, we explored the sub-cellular localization of L mutant viruses by immunofluorescence analysis in LF-BK infected cells (Fig. 6). Very early during infection (2 hpi), there was an L positive signal in WT virus infected cells when no VP1 signal could still be detected. By 3 hpi, the L signal was mainly localized to the nuclei of infected cells, while VP1 was detected in the cytoplasm; thereafter the L signal was detected throughout the whole cell, as previously described for FMDV type A12 (de los Santos et al., 2007, 2009). The pattern of L sub-cellular localization of the L mutant viruses was similar to the WT virus, except for a delay in the cytoplasmic L protein signal. These results demonstrated that the extended Lb protein in mutant A24-L1123/Flag did not affect the functions or cellular localization of L in cell culture.

4. Discussion

The FMDV leader proteinase exists in two forms, termed Lab and Lb, which exhibit similar proteolytic activities (Cao et al., 1995; Medina et al., 1993; Strebel et al., 1986). This conclusion is based on *in vitro* studies that used plasmids expressing each individual form of L. However, it remains unknown whether each form of L plays a distinctive role in viral replication and pathogenesis. Because Lab and Lb only differ at their amino terminus, a polyclonal anti-L antiserum cannot differentiate between them. Moreover, the hydrophobic character of this region makes difficult to generate a specific antiserum against it. Therefore in this study, we introduced a tag (HA, Flag or tc) in a mutant virus (A24-L1123) that already contains a tn insertion of 57 nt extras in the inter-AUG region and analyzed the functions of the resulting tagged Lab protein on viral translation and replication.

In vitro translation assays showed that the processing of L was unaffected by the presence of the N-terminal tags, however we only recovered infectious A24-L1123/Flag and A24-L1123/tc mutant viruses. These mutant viruses showed small plaque phenotype compared to the WT virus in cell cultures similarly to the parental mutant virus A24-L1123. However, we could not detect the expression of the flag or tc tags in the recovered mutant viruses. Interestingly, mutant virus A24-L1123/Flag acquired an extra base in the inter-AUG region that resulted in new AUG codons in-frame with the second AUG codon. This mutation restored the 75 nt distance between initiation codons that occurs naturally in FMDV serotypes SAT 1–3. Western blot analysis of A24-L1123/Flag infected cell extracts revealed a unique L protein slightly bigger than Lb, consistent with translation initiation from these AUGs. Remarkably, this extended Lb protein performed as well as the WT Lb for all known functions of L in cell culture. This result is consistent with the fact that the N-terminus of the Lab protein is highly variable among all FMDV serotypes (George et al., 2001; Tosh et al., 2004). In the case of mutant virus A24-L1123/tc, western blot analysis showed a prominent band for Lb and a fainter slower band, presumably tc tagged L protein. Unfortunately, we could not visualize the presence of the tc tag in this species, probably because there was not enough protein to be detected with the available fluorescent reagents (data not shown).

These results are consistent with previous studies by Cao et al. (1995) where utilizing another approach that consisted in generating mutant viruses that encoded only one form of the L protein; could not produce mutant virus encoding for the Lab protein. Although we failed to visualize the Lab protein, in this study we derived several mutant FMD viruses that allowed us to explore the role of the inter-AUG region in viral translation.

Most studies concerning the involvement of inter-AUG region in translation use *in vitro* systems with bicistronic expression plasmid constructs (Belsham, 1992; Lopez de Quinto and Martinez-Salas, 1999; Ohlmann and Jackson, 1999; Poyry et al., 2001). In a previous study in cell culture, we demonstrated that the inter-AUG region is essential for virus replication since viable L-deleted virus could only be obtained when this region was maintained and translation of the viral polyprotein started from the second AUG codon (Piccone et al., 1995a).

Initiation of translation can occur at more than one AUG codon in some picornavirus (Hinton et al., 2000; Hollister et al., 2008; Kaminski et al., 1994; Kong and Roos, 1991; Tesar et al., 1992) by a poorly understood mechanism of selection. The selection of the correct initiation codon is critical, since incorrect AUG selection could lead to miscoded or truncated proteins. In the case of FMDV, it has been proposed that initiation occurs at the second AUG by a mechanism similar to the ribosome scanning model of Kozak (1989). The recognition of the correct initiation codon during this process has been shown to be inhibited by RNA structures and by the presence of additional AUG codons (Belsham, 1992; Jackson and Kaminski, 1995). Alternatively, Andreev et al. (2007) have hypothesized that selection of the second AUG occurs independently of the recognition of the first AUG codon. Interestingly, the tn insertion in our mutant viruses creates a stem-loop structure with a ΔG of −43.5 kcal/mol according to the mfold program (Zuker, 2003). Therefore, according to the scanning model, the introduction of the tn should have inhibited the initiation from the second AUG. However, under the experimental conditions assayed in Fig. 4, the tn did not have any effect on the initiation from the Lb site in mutant viruses A24-L1123 or A24-L1123/tc. Nevertheless, it is possible that the presence of the tn slowed down the scanning of the 40S ribosomal complex, thereby allowing internal initiation from the second

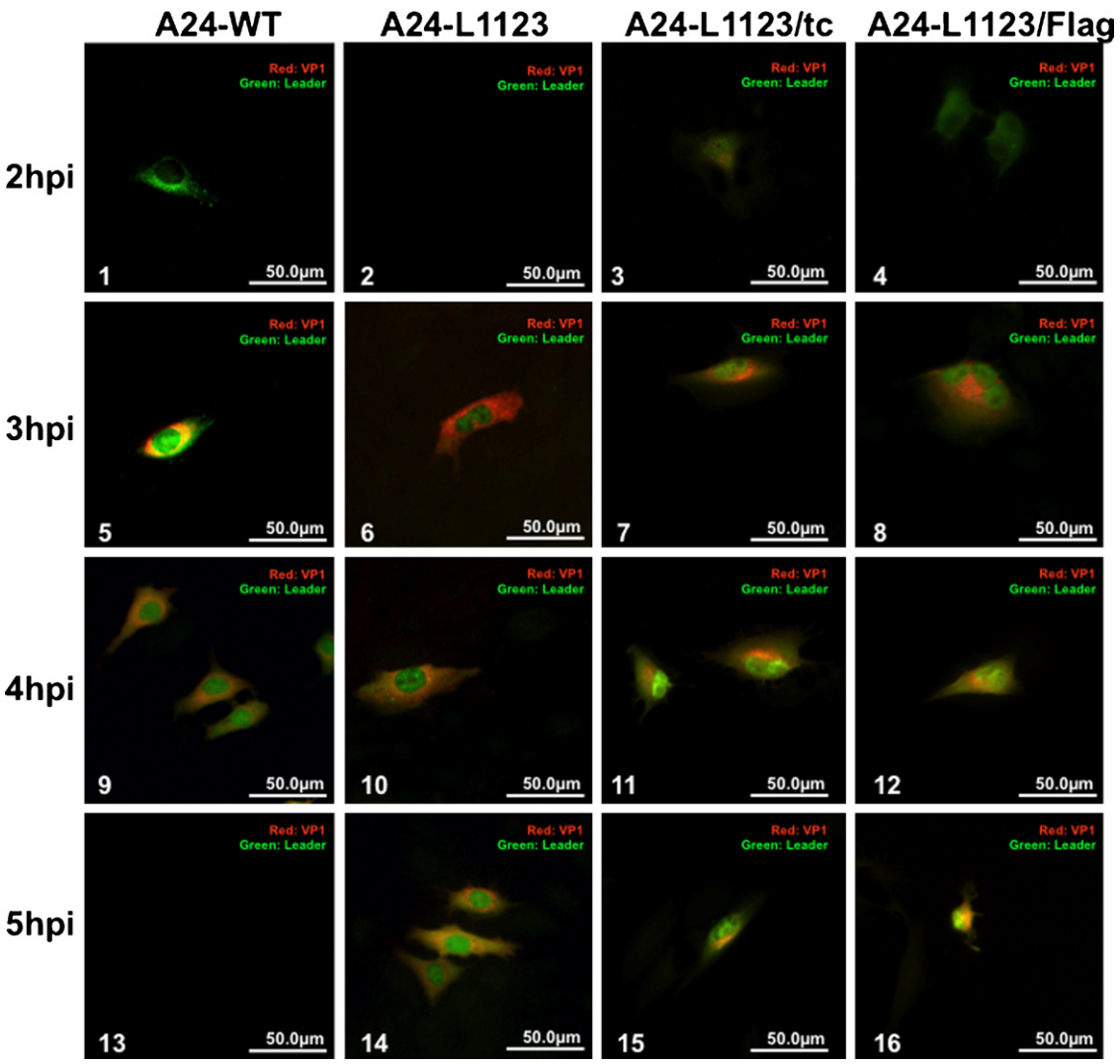

Fig. 6. Localization of leader in WT and L-mutant viruses in infected cells. LF-BK cells were infected with A24-WT (panels 1, 5, 9 and 13), A24-L1123 (panels 2, 6, 10 and 14), A24-L1123/tc (panels 3, 7, 11 and 15) or A24-L1123/Flag (panels 4, 8, 12 and 16) at MOI 10. At given times post-infection, cells were fixed with 4% paraformaldehyde and stained with anti-leader and anti-VP1 antibodies. Leader was detected with Alexa fluor 488 (green) and VP1 with Alexa fluor 594 (red) conjugated secondary antibodies.

AUG codon. Slowed viral translation could explain the attenuated phenotype observed by the tn-L mutant viruses, as shown in Fig. 5 by the delayed virus growth in cell culture. Decrease virus load could give the host enough time to develop an antiviral response and control infection. In addition to the proposed structure, the insertion of the tn increased the distance between both AUGs to 141–168 nt. However, such an increase in the length of the inter-AUG region had minimum effect on the initiation of translation from the second AUG. Similar results were reported by Lopez de Quinto and Martinez-Salas (1998).

Overall, the data shown here suggest that attenuation of the tn-L mutant viruses does not to correlate with the amino acid sequence of the N-terminus of the L protein, but probably with the structure of the inter-AUG region. It is, however, possible that the tn insertion interferes with binding of cellular factors required for efficient translation. Further mutational analysis will determine the biological role of the inter-AUG region of FMDV in the viral cycle.

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